



Early Journal Content on JSTOR, Free to Anyone in the World

This article is one of nearly 500,000 scholarly works digitized and made freely available to everyone in the world by JSTOR.

Known as the Early Journal Content, this set of works include research articles, news, letters, and other writings published in more than 200 of the oldest leading academic journals. The works date from the mid-seventeenth to the early twentieth centuries.

We encourage people to read and share the Early Journal Content openly and to tell others that this resource exists. People may post this content online or redistribute in any way for non-commercial purposes.

Read more about Early Journal Content at <http://about.jstor.org/participate-jstor/individuals/early-journal-content>.

JSTOR is a digital library of academic journals, books, and primary source objects. JSTOR helps people discover, use, and build upon a wide range of content through a powerful research and teaching platform, and preserves this content for future generations. JSTOR is part of ITHAKA, a not-for-profit organization that also includes Ithaka S+R and Portico. For more information about JSTOR, please contact support@jstor.org.

A NEW METHOD FOR DIFFERENTIAL STAINING OF BACTERIA

F. M. SCALES

From the Office of Soil Bacteriology Investigations, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C.

In 1884, Christian Gram¹ while working with sections of nephritic material, discovered that if he stained the tissue with Ehrlich's anilin gentian violet solution and then with Lugol's solution a subsequent treatment with alcohol decolorized the tissue but did not remove the stain from some pathogenic bacteria in it. Further tests by Gram and others showed that the procedure could be used to divide the family of bacteria into two great groups: those that retained the stain and those that were decolorized when treated with alcohol. The method proposed by him has been closely followed in theory ever since and has gained universal application in bacteriology on account of the diagnostic value of the data obtained from it. The changes in method that have been suggested from time to time have been confined, for the most part, to various ways of preparing the violet solution in order to obtain one of better keeping quality. Recently Burke² has described a method for the Gram stain in which he has recommended the use of acetone or acetone and ether for the decolorizer.

The procedure reported here, while differentiating the same groups of organisms as Gram's method, does not follow the theory of his work, since, along with other differences, the iodine solution is not used.

METHOD

Solutions: The stain used is cotton blue C₄B (Poirrier's Blue)³ which is obtained by the action of sulphuric acid on triphenylrosanilin.

Received for publication, Aug. 14, 1922.

¹ Fortschritte der Medicin, 1884, 2, p. 185.

² Jour. Bacteriol., 1922, 7, p. 159.

³ The cotton blue C₄B may be obtained from Walter F. Sykes & Company, 8 Lispenard Street, New York. This firm is the American representative of Societe Anonyme des Matieres Colorantes et Produits Chimiques de Saint-Denis.

One sample of an American made cotton blue C₄B was tested but did not give satisfactory results. The dye became a reddish purple in some of the positive cultures after counter-staining, and when a small amount of acid was added to the solution to make the blue persist the negative cultures did not decolorize properly. It did not seem worth while to work further with the American dye at present because no doubt the process for making it will be improved so that a dye comparable to that originally patented by Poirrier will be produced. In this connection, it is interesting to note that the present imported sample is exactly the same in reaction as a nine-year old one with which this work was started.

One gram of the dye is placed in a mortar, moistened with 1 c.c. of 95% alcohol and then stirred and ground with a glass stirring rod for a couple of minutes, until the mixture becomes a thick, smooth liquid. One hundred c.c. of an aqueous phenol solution are added and the grinding continued for a minute or two until all lumps of dye have dissolved, when the solution is ready for use. The phenol solution is prepared by adding 5 c.c. of the melted phenol crystals to 100 c.c. of distilled water, shaking vigorously and then filtering.

The second solution is a combined decolorizing and counterstaining agent. It is prepared by dissolving 2 gm. of safranin⁴ in 100 c.c. of 95% alcohol and then adding 100 c.c. of acetone c. p.⁵ The alcoholic soluble safranin was used but a test with a water soluble sample gave equally good results.

Procedure.—The bacterial film is spread in the usual way. The customary precaution should be taken not to make it too heavy. If allowed to dry on the slide no fixing is necessary, but the drying and fixing may be hastened by slightly heating if desired. The film is then covered with a few drops of the blue solution for from 20 to 30 seconds. The excess stain is drained off and the slide well washed in running water. The stain from the slide may be saved and used repeatedly. A couple of drops of the safranin solution are allowed to run down the washed slide to remove the excess water and the preparation then covered with the safranin solution for 3 or 4 minutes. The slide can be set down during this time as it is not necessary to tilt it back and forth to obtain a clearing of negative organisms. It is better practice to decolorize and counterstain in a Coplin jar.⁶ The slide may now be washed in running water and when dry is ready for examination.

The gram-positive organisms are colored a very deep blue and the negative ones the red characteristic of safranin.

Two species of micrococci tested with this method gave some blue cells and the rest dark purple when decolorized and counterstained for 3 minutes. In order to obtain a larger number showing characteristic blue staining the cotton blue C₄B solution was left on the film for 1 minute and the decolorizing and counterstaining continued for 4 minutes. This change gave the desired result and a smear of *B. coli* on the same slide was stained red.

RESULTS

The procedure outlined was used in a test on 16 cultures of bacteria that had also been stained with Ehrlich's anilin gentian violet solution in the usual way for Gram staining. In every instance the proposed method gave the same positive and negative results that were obtained with the anilin gentian violet stain.

The organisms⁶ employed for this work were:

⁴ Safranins obtained from Sykes & Company, Coleman and Bell (safranin A conc.), Shoemaker and Busch, and the Will Corporation gave satisfactory results.

⁵ On a very warm day during which there was considerable evaporation the acetone-alcohol solution volatilized so fast that a slide became almost dry and the solution so concentrated that the positive organisms were decolorized and counterstained. This was the only time, however, when the usual method of applying the stain failed to give satisfactory results. A duplicate slide placed in a Coplin jar was properly decolorized and counterstained at this time.

⁶ For some of these cultures the author is indebted to Messrs. N. R. Smith and L. T. Leonard of this office, to Dr. J. M. Sherman of the Dairy Division, Bureau of Animal Industry, U. S. D. A., and to Dr. E. Lefevre, Microbiological Laboratory, Bureau of Chemistry, U. S. D. A.

Positive	Negative
<i>B. asterosporus</i>	<i>B. coli</i>
<i>B. cereus</i>	<i>B. fluorescens</i>
<i>B. megatherium</i>	<i>B. prodigiosus</i>
<i>B. simplex</i>	<i>B. radicicola</i> (red clover)
<i>B. subtilis</i>	<i>B. radicicola</i> (vetch)
<i>B. vulgatus</i>	<i>Ps. pyocyanea</i>
<i>Bact. mycoides</i>	
<i>M. pyogenes</i> var. <i>aureus</i>	
<i>M. candidus</i>	
<i>Mycobact. diphtheriae</i>	

THE THEORY

The phenomenon associated with the retention of the violet stain in the Gram method has been explained as due to purely chemical reactions by Unna⁷ and to physical ones by Brudny.⁸ Benians,⁹ in a series of experiments to test the basis of these explanations, while not wholly agreeing with Brudny, does come to the conclusion that the reaction is a physical one. Previous investigators in seeking a solution of this problem have been under the disadvantage of having to precipitate the violet stain within the cell before they could test for decolorization. In the procedure presented here that difficulty is removed, and so the explanation is to that extent simplified.

The imported cotton blue C_4B when made up in aqueous solution and employed in the usual way is retained¹⁰ by a few cells in a positive culture although the great majority when counterstained are red or purple. When the stain is made up in the phenol solution all the cells are blue after counterstaining. The stain is soluble in phenol, which, possessing a great affinity for living matter, thus fixes the dye to the cell substance. This fixing power is due to a physicochemical reaction.

The data obtained indicate that physical phenomena control the results in the remainder of the procedure. If 1.5 gm. of boric acid¹¹ are dissolved in 100 c c of the decolorizing and counterstaining reagent, to increase the solution pressure, and films of different cultures stained with cotton blue C_4B , then treated with it for 6 minutes, a large number of cells in certain weakly gram-positive cultures show a negative staining

⁷ Monatschr. f. prakt. Dermat., *Erganzungsheft*, 1887.

⁸ Centralbl. f. Bakteriöl. u. Parasitenk., II, 1908, 21, p. 62.

⁹ Jour. Path. & Bacteriol., 1912-13, 17, p. 199; 1920, 23, p. 401.

¹⁰ In the usual Gram staining method this is recognized as a characteristic of dyes of the pararosanilin group.

¹¹ Sodium acetate or ammonium nitrate will give the same result.

while films of the same cultures, if dried on a double filter paper for 1 minute before immersion in the decolorizing and counterstaining solution, show practically all positive cells. The strongly positive species remain blue under both treatments. Further tests may show that a definite grouping of strongly and weakly positive species may be obtained by a comparison of results on moist and dried slides with this decolorizing and counterstaining solution containing boric acid. *B. coli* is negative, except possibly for a blue ring around the edge of the smear where it is thickest, under this treatment when decolorized and counterstained for 6 minutes; but if the time of decolorization is limited to 3 minutes, the moist films are negative and the dried ones positive.

Strongly positive species may be made negative by covering a stained film of such a culture with the regular decolorizing and counterstaining solution and then gently heating on a hot plate for 5 minutes. A somewhat less complete decolorization and counterstaining may be obtained by immersing in the regular safranin solution to which 8 gm. of ammonium nitrate per 100 c c have been added. At the end of 2 hours the slide is placed in distilled water for 20 minutes and then returned to the solution mentioned in the foregoing for about 3 hours, when it is placed in the safranin solution containing boric acid for a short time. This procedure yields a majority of pale pink rods with some pinkish purple and some blue ones.

Acetone acts as a dehydrating agent only, and by removing the water, as drying with filter paper does, yields a larger number of blue cells in weakly positive cultures. If the decolorizing and counterstaining solution contains only 25% or less of acetone, more red and purple cells are found in such cultures, showing that the dehydration has not been sufficient to reduce the speed of osmosis.

All these facts indicate that the phenomenon depends on the permeability of the cell sheath and that as the water within the cell is reduced osmosis is correspondingly slowed up so that a much longer time is required to decolorize it.

DISCUSSION

The addition of a small amount of alcohol is advised for the preparation of the solution of cotton blue C_4B , because some of the stain dissolves in it and the remainder in this case goes into solution more readily in the phenol water. The solution prepared in this way seems to keep indefinitely, as one made eight months ago shows no change and stains as well now as when first tried. The only precaution taken to preserve it was to keep the bottle tightly stoppered.

It may not be amiss here to call attention to the fact that safranin is better for this procedure than other red dyes because it does not overstain if left on a smear longer than usual.

It hardly seems necessary to point out the advantages of a procedure which entirely eliminates the use of one solution that has always been considered necessary, and which also provides for simultaneous decolorizing and counterstaining of bacterial films. This method also gives the bacteriologist an opportunity to adopt a uniform time for decolorizing preparations.

The organisms used in this procedure were stained when they were 1 and also 3 days old. The results were the same in both instances.

The slides showing the greatest contrast were obtained by staining and decolorizing for the longer periods, namely, 30 seconds and 4 minutes. Some of the species stained with the blue for 5 to 10 seconds and then decolorized and counterstained for 45 to 60 seconds showed characteristic coloration; but since other species gave better results with slightly longer staining, the longer time is recommended.

SUMMARY

A new method is presented for the differential staining of bacteria. It yields results like those obtained with Gram's procedure.

A cotton blue C₄B solution is employed for the initial staining.

No iodine is used.

Decolorizing and counterstaining are done with one solution.

When tested with a decolorizing and counterstaining solution containing boric acid, sodium acetate or ammonium nitrate, a large number of cells in weakly gram-positive cultures were negative; but when a film of the same culture was dried on filter paper nearly all the cells were strongly positive.

A film of *B. coli* was positive on a dried slide that was decolorized and counterstained (solution containing boric acid) for the same length of time that gave a negative staining on a moist one.

Species that are strongly positive become negative when the film of such a culture covered with the regular decolorizing and counterstaining reagent is gently heated for five minutes. A less complete decolorization and counterstaining may be obtained by immersing for a long time in the regular solution to which an excess of ammonium nitrate has been added.

The reaction is a physicochemical one depending on the affinity of the dye for cell substance, supplemented by the fixing power of phenol, and the permeability of the cell sheath.